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Abstract

The *MET* oncogene, a crucial regulator of the genetic program known as “invasive growth” or “epithelial–mesenchymal transition,” has recently emerged as a functional marker of glioblastoma stem cells. Here, we review findings that associate MET expression and activity with a specific, genetically defined glioblastoma stem cell subtype, and data showing how MET sustains the stem cell phenotype in glioblastoma and other tumors. Finally, we discuss issues related to identification of tumorigenic clones driven by MET in the context of genetically heterogeneous tumors and strategies aimed at eradicating cancer stem cells.

Glioblastoma: A Model for the Quest of Tumor Genetic Determinants and Tumorigenic Cell Hierarchies

Glioblastoma, the most common type of brain tumor, is relatively rare but virtually incurable, with a median survival of 12 to 15 months attained through combination of surgery, radiotherapy, and chemotherapy with the DNA alkylating/methylating agent temozolomide (1). This multimodal protocol often fails because the locally infiltrative nature of the tumor may limit surgical resection, brain side effects restrain the dose of radiotherapy, expression of the DNA repair enzyme O-6 methyl-guanine methyltransferase (MGMT) confers resistance against temozolomide, and the blood–brain barrier blocks alternative chemotherapeutic agents (2, 3). With the exception of antiangiogenic agents, inhibitors of tyrosine kinase receptors, or signal transducers controlling cell proliferation, did not provide any benefit in clinical trials (4). Recurrence has been associated with innate DNA repair activity and radioresistance of the “cancer stem cell” subpopulation (5). Glioblastoma is indeed one of the first solid tumors in which a cell hierarchy including tumorigenic (stem) and nontumorigenic (nonstem) cancer cells has been identified by the *in vivo* transplantation assay (6, 7). At the same time, a comprehensive picture of the glioblastoma genomic landscape has been provided (reviewed in ref. 8). Genes recurrently altered—and thus likely to play a significant pathogenic role—frequently impinge on three main signaling circuits: (i) the receptor tyrosine kinase/Ras/phosphoinositide 3-kinase (PI3K) pathway, controlling cell proliferation and survival [This includes the EGF receptor (*EGFR*, amplified and/or mutated in 45% cases, the most frequent mutation being deletion of the extracellular domain, also known as *EGFRvIII* deletion). Other affected genes are the PI3K inhibitor *PTEN*(inactivated in 36% of cases) and the Ras inhibitor *NF1* (inactivated in 23% of cases)]; (ii) the p53 pathway, controlling apoptosis and senescence, where *TP53* is mutated in 35% of cases; and (iii) the RB pathway, controlling cell-cycle progression, where the cell-cycle inhibitors *CDKN2A* (p16/INK4A) and *CDKN2B* are alternatively inactivated in about 50% of cases. Overall, about 75% of patients harbor at least one gene alteration in each of the above three pathways (2, 8). Another recently emerged pathway is NF-ºB, with deletion of*NFºBIA* affecting about 25% of patients (9). Quite unexpectedly, a relatively high frequency of mutations was also found in the Krebs cycle enzymes isocitrate dehydrogenase 1 and 2 (*IDH1/2*). These mutations are preferentially associated with “secondary glioblastoma,” that is, glioblastoma progressing from lower grade gliomas, where they affect 80% of cases, with a significant pathogenetic role (10). As the inventory of glioblastoma-driving genes enlarges, the connectivity among the crucial players is becoming clearer. However, the dynamics by which mutations accumulate is still obscure, particularly in “primary glioblastomas,” that is, tumors diagnosed at their onset as “high-grade” (10). Intratumor genetic heterogeneity—the presence of different genetic lesions in distinct cell subsets—is a common cancer feature, and glioblastoma makes no exception

(reviewed in ref. 11). Intriguingly, this heterogeneity might reflect coexistence of subclones sustained by genetically distinct cancer stem cells. Indeed, it has been proposed that cancer stem cells can be not only a source of tumor phenotypic heterogeneity (resulting from progeny pseudodifferentiation) but also the units of tumor genetic evolution, obeying the Darwinian laws of mutation and selection (12, 13). Studies in leukemias have shown that multiple, genetically distinct subclones of cancer stem cells may coexist (14). These sublcones likely result from "divergent evolution" of a common ancestor cancer stem cell, which generates parallel lineages undergoing independent mutation accrual and clonal selection (12–14). Interestingly, genetically distinct, but related, cancer stem cells have been isolated from different areas of the same glioblastoma (15), suggesting that, also in this tumor, divergent evolution and coexistence of different cancer stem cell subclones may occur, although a dominant subclone may prevail.

Concomitant analysis of genetic alterations and gene expression profiles of glioblastomas provided a molecular classification into four main subtypes (proneural, neural, classical, and mesenchymal; ref. 16). In this classification, some genetic alterations are preferentially associated with a specific gene expression profile. For instance, high-level amplification and/or mutation of *EGFR* gene, together with high expression of EGFR protein, are significantly more frequent in tumors displaying the classical or the neural profile, as compared with the proneural or mesenchymal ones. Platelet-derived growth factor (*PDGF*) receptor or *IDH1/2* alterations define the proneural profile, whereas *NF1*deletion is mostly associated with the mesenchymal. Conversely, another frequent gene alteration such as *PTEN* inactivation may associate with any gene expression profile (16). Interestingly, glioblastomas that relapse after undergoing the selective pressure of chemoradiotherapy usually display a mesenchymal profile (16). This suggests that, in the primary tumor, a mesenchymal subclone inherently resistant to therapies may coexist with a dominant classical or proneural subclone and drive relapse when such dominant clone is exterminated.

The MET Oncogene: A Marker of a Glioblastoma Stem Cell Subset

Beside *EGFR*, other receptor tyrosine kinase genes are altered in a significant fraction of glioblastomas: *HER-2* (*ERBB2*, 8%), *PDGF* receptor A (13%), and *MET* (HGF receptor, 4%). Although its genetic alteration is relatively rare, *MET* is suspected to play a wide role in glioblastoma pathogenesis, as it is often overexpressed and coexpressed with its ligand, hepatocyte growth factor (HGF; refs. 17–19). The frequency of MET expression in primary glioblastoma, measured by immunohistochemical methods, varies from 100% (likely overestimated because of the different levels of intensity among samples; ref. 17) to approximately 30% of cases (20). Interestingly, MET is expressed also by endothelial cells and can significantly contribute to glioblastoma neoangiogenesis (19). Through gene expression profiling, *MET* turned out to be a "signature gene," specifically associated with the glioblastoma mesenchymal subtype (30% of primary glioblastomas; refs. 16, 21).

The study of MET expression and function, as well as other tyrosine kinase receptors, in glioblastoma stem cells has been facilitated by *in vitro* isolation and long-term propagation of glioblastoma neurospheres (hereafter referred to simply as "neurospheres"), that is, extensively self-renewing cultures enriched in glioblastoma stem and progenitor cells (7). In neurospheres, both EGFR and MET were shown to sustain the stem and tumorigenic phenotype (22, 23). More recently, MET expression was associated with glioblastoma stem cells identified by prospective isolation from fresh tumors (24) or with neurospheres endowed with specific genetic/molecular features (25). The study by Joo and colleagues shows that MET expression is heterogeneous within the same tumor, with dominant expression in two apparently unrelated regions: the proximities of blood vessels and the hypoxic edges (24). The first region may correspond to the "perivascular niche," a microenvironment required to maintain glioblastoma stem cell properties (26). Hypoxic areas, which are far from blood vessels and adjacent to necrotic areas, have been already functionally associated with MET in many tumors, since a transcriptional factor activated by hypoxia [hypoxia-inducible factor 1 (HIF-1)] directly promotes transcription of the *MET*gene (27). Prospective isolation of glioblastoma cell subpopulations with anti-MET antibodies showed that only cells expressing high levels of MET retained clonogenic, tumorigenic, and radioresistant properties—that is, a cancer stem cell phenotype (ref. 24;Fig. 1).

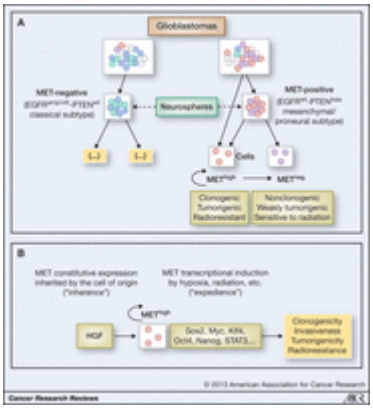

Figure 1.

MET as a functional marker of glioblastoma stem cells. A, from glioblastomas, two subtypes of neurospheres (cultures enriched in stem and progenitor cells) can be isolated that alternatively lack or display MET expression (MET-negative or MET-positive, respectively). Lack of MET expression significantly correlates with *EGFR* gene amplification/mutation ($EGFR^{amp/vIII}$), "wild-type" *PTEN*gene ($PTEN^{wt}$), and a classical gene expression profile (subtype). Conversely, MET expression preferentially associates with a "wild-type" *EGFR*gene ($EGFR^{wt}$), *PTEN* gene inactivation ($PTEN^{loss}$), and a mesenchymal or proneural gene expression profile. In MET-positive neurospheres, a cell hierarchy can be found. The MET^{high} subpopulation displays clonogenic and tumorigenic properties and radioresistance. MET^{high} cells self-renew and generate cells that downregulate MET expression (MET^{neg}) and lose stem/tumorigenic properties. MET^{high} cells can also be directly isolated from glioblastoma tissues. The cell hierarchy in MET-negative neurospheres is presently unknown. B, MET expression may be constitutive ("inherence," see text) but also increased by environmental factors ("expedience," see text). In MET^{high} cells, HGF drives a genetic program that sustains clonogenicity, invasiveness, tumorigenesis, and radioresistance.

The study by De Bacco and colleagues associated MET expression with a subset of neurospheres endowed with specific genetic features (25). MET is usually absent from neurospheres displaying *EGFR* amplification/mutation (MET-negative neurospheres, Fig. 1). Conversely, MET is usually expressed in neurospheres harboring a normal *EGFR*gene and *PTEN* inactivation (MET-positive neurospheres, Fig. 1). A corollary observation of this study is that while in the tumor tissue *EGFR* amplification/mutation ($EGFR^{amp/vIII}$) and *PTEN* inactivation ($PTEN^{loss}$) often coexist, in neurospheres the two genetic lesions dissociate (Fig. 1). Interestingly, in the same study, a subgroup of original tumors harboring both $EGFR^{amp/vIII}$ and $PTEN^{loss}$ yielded neurospheres with normal *EGFR* gene ($EGFR^{wt}$) and $PTEN^{loss}$ (25). These observations, although based on a small number of cases, suggest that (i) glioblastomas harboring both $EGFR^{amp/vIII}$ and $PTEN^{loss}$ contain at least two subclones of cancer stem cells, respectively, $PTEN^{loss}$-$EGFR^{wt}$ and$PTEN^{loss}$-$EGFR^{amp/vIII}$; (ii) from the same tumors, *in vitro* culture positively selects$PTEN^{loss}$-$EGFR^{wt}$ subclones; and (iii) in glioblastoma progression, *PTEN* inactivation should occur before *EGFR* amplification/mutation, a conclusion sustained also by predictive mathematical models (28).

MET expression was also associated with defined neurosphere gene expression profiles (25). Classification on the basis of the signatures identified by Verhaak and colleagues in glioblastoma tissues (16) indicated that MET-positive neurospheres mostly belonged to the mesenchymal subtype, an association already observed in glioblastoma tissues (16,21). However, quite unexpectedly, some Met-positive neurospheres were classified as proneural. Conversely, MET-negative neurospheres mostly belonged to the classical subtype, thus conserving the same relationship between *EGFR* gene amplification/mutation and classical gene expression profile observed in glioblastoma tissues (Fig. 1).

MET Sustains the Stem, Tumorigenic, and Invasive Phenotype in Glioblastoma

It is well known that the HGF/MET ligand–receptor pair plays a functional role in glioblastoma as well as in other cancer cell lines by driving the "invasive growth" program. This is a complex biologic process starting with epithelial–mesenchymal transition (EMT) and leading to cell invasion, proliferation, and survival in atypical tissue environments, including distant metastatic sites (reviewed in ref. 29). Recent evidence indicating that common molecular mechanisms control both EMT and "stemness" (30) suggests that MET may be involved in the concomitant regulation of both properties.

Three recently published articles support the conclusion that MET is a functional marker of glioblastoma stem cells. Li and colleagues showed that MET stimulation by HGF sustains clonogenic properties of neurospheres and expression of a panel of transcription factors, including Sox2, c-Myc, Klf4, Oct4, and Nanog, capable of reprogramming differentiated cells into pluripotent stem cells (ref. 23; Fig. 1). The studies by Joo and colleagues (24) and De Bacco and colleagues (25) corroborate the ability of MET to sustain the

glioblastoma stem cell phenotype *in vitro* and report a tight association between MET expression and tumorigenic properties *in vivo*. However, MET is not a universal marker of glioblastoma stem cells. Indeed, MET-negative neurospheres fully endowed with self-propagation and tumorigenic properties can be derived, mostly from glioblastomas harboring *EGFR* gene amplification/mutation (Fig. 1). Unlike MET-positive neurospheres, MET-negative neurospheres are strongly dependent on EGFR activation for their *in vitro* propagation and, likely, for their *in vivo* tumorigenic potential (25).

Interestingly, MET-positive neurospheres do not homogenously express MET and seem to be organized in a cell hierarchy featuring, at the apex, a cell subpopulation expressing high levels of MET (MET^{high}) and, at the base, a cell subpopulation that conversely expresses negligible levels of MET (MET^{neg}; Fig. 1). This conclusion is supported by data showing that upon isolation from neurospheres, (i) MET^{high}, but not MET^{neg} cells, retain distinctive stem cell properties such as long-term propagation (clonogenic ability) and multipotential differentiation; (ii) MET^{high}, but not MET^{neg} cells, can reconstitute a mixed MET^{high}-MET^{neg} cell population, as found in the original neurosphere; and (iii) MET^{high}have increased tumorigenic ability as compared with MET^{neg} and are the only that form tumors containing both MET^{high} and MET^{neg} cells (25). Moreover, it was shown that when neurospheres undergo a differentiative program (e.g., by culture in serum) MET expression is downregulated, again suggesting that MET is specifically associated with the stem status (25).

Finally, in MET-positive neurospheres, HGF increases *in vitro* migration through extracellular matrices, a typical property of "mesenchymal" cells, predictive of invasive ability *in vivo*, thus suggesting that MET may concomitantly regulate stem and EMT/invasiveness (Fig. 1; ref. 25).

MET Expression and Function in Cancer Stem Cells: A Paradigm of "Inherence"

Together with leukemias, glioblastoma is a model for the genetic and phenotypic study of cancer stem cells in many tumor types. High incidence tumors, such as breast, lung, and colorectal carcinomas, underwent extensive genetic characterization, but investigation on how mutations affect the cancer stem cell phenotype has just begun. With the advent of targeted therapies, this knowledge is necessary to clarify: (i) whether a given target is expressed and functionally meaningful in the dominant stem cell subpopulation, which would determine whether the arising tumor will be sensitive or primarily resistant to target inhibition; and (ii) whether, in the same tumor, there are coexisting cell subclones with stem potential primarily resistant to, and therefore selectable by, therapy to become the driver of tumor recurrence. This is exemplified by a minor subset of *MET*-amplified cells resistant to EGFR inhibitors, which drives recurrence in non–small cell lung carcinoma after treatment with gefitinib (31).

There is little knowledge about expression and function of MET in cancer stem cells outside the brain; however, direct and indirect evidence points to an ample involvement in defining tumorigenic subpopulations. In colorectal cancer, this oncogene is frequently overexpressed, mostly in association with poor prognosis (32). It has been shown that colorectal cancer stem cells express MET, activated by the HGF abundantly secreted by tumor-associated fibroblasts, which supports the stem phenotype by sustaining the Wnt self-renewal pathway (33). An indication that MET plays a role in breast cancer stem cells comes from the study of cell hierarchies in the mouse mammary gland: Here, MET is specifically expressed in the "luminal progenitor" subpopulation (34), a likely origin of the basal-like cancer subtype (35), where the oncogene is frequently overexpressed (36). Indeed, in many tumors, the *MET* gene is rarely mutated (or amplified) but often overexpressed in the absence of genetic alterations (reviewed in refs. 32, 37). Recently, we proposed that this overexpression is an adaptive response to a threat, providing a mechanism of escape from danger, and an oncogenic boost ("oncogene expedience;" ref.32). Hypoxia, inflammatory cues, and ionizing radiation are among the adverse environmental conditions leading to activation of specific signaling pathway and transcriptional mechanisms (HIF, NF-ºB) that upregulate *MET*. The oncogene, in turn, unleashes a powerful prosurvival signal and, eventually, escape from the "mined" territory through invasion and metastasis (27, 38).

We now propose that, in addition to expedience, MET overexpression by tumors is a paradigm of "inherence." This means innate MET expression by cancer stem cells that "inherit" MET as a physiologic trait from their normal counterparts, that is, the stem and progenitor cells that practice invasive growth as part of their normal phenotype (39). In this context, MET overexpression in tumors is due not only to transcriptional induction at single-cell level but also to expansion of the stem/progenitor subpopulation of cells inherently

expressing MET (39). Indeed, MET expression and activity has been shown to support amplification and self-renewal of murine adult neural stem cells of the subventricular zone (40), the most likely, although not the only possible, glioblastoma cells of origin (10). Expansion of the cell compartment expressing MET is consistent with accumulation of stem/progenitors resulting from the differentiation block imposed by oncogenic transformation. This block may worsen during tumor progression and possibly lead to the equivalent of a leukemic "blastic crisis."

Implications of MET Expression in Cancer Stem Cells for Diagnosis and Personalized Therapy of Glioblastoma (and Other Tumors)

The presence of different mutations in cancer stem cells, their accumulation over time in linear or in branching subclones, and the likely coexistence of genetically distinct subclones has important consequences: (i) the inter- and intratumor variegate genetics of cancer stem cells undermines the response to targeted therapies; (ii) the intratumor variability of cancer stem cells dictates the need to hit simultaneously different coexisting subclones; and (iii) the survival of any minor clone—unaffected by therapy—drives positive selection and recurrence.

A trivial explanation for failures of clinical trials with targeted therapies in glioblastoma may be the presence of the blood–brain barrier that opposes an obstacle to drug delivery. However, in glioblastoma as well as in other tumors not shielded by a blood–tissue barrier, a deeper interpretation of these failures is the heterogeneous and dynamic genetics of cancer stem cells. In glioblastoma, the high frequency of *EGFR*amplification/mutation fostered EGFR inhibitors. Despite initial success in patients displaying specific molecular features, such as expression of *EGFRvIII* and *PTEN*integrity (41), subsequent trials failed (42). As result, therapy of glioblastoma with EGFR inhibitors was stopped. However, from these studies, it is difficult to draw a final negative conclusion, as a different outcome could arise from careful stratification of patients by considering the full spectrum of genetic alterations and recognizing the presence of coexisting subclones harboring genetic lesions other than *EGFR*. Similar considerations must be taken into account in planning "personalized" targeted therapy against MET. As mentioned above, the oncogene is amplified only in a small subset of glioblastomas (4%), but it is expressed in a high percentage of cases, often in association with its ligand HGF, which is predictive of sensitivity to MET inhibition in mouse models (43). Moreover, as discussed, MET may contribute to sustain the stem cell phenotype of a fraction of glioblastomas by "oncogenic inherence" (see above). However, as suggested also by a phase II clinical trial with the anti-HGF antibody AMG-102 (44), it is unlikely that MET targeting alone could provide a substantial benefit in glioblastoma therapy. On the other hand, preclinical studies have shown that MET inhibitors synergize with EGFR against xenografts of glioblastoma cell lines harboring both *EGRvIII* mutation and *PTEN* deletion (45). Therefore, better identification of patients that will benefit from MET inhibitors could be achieved by (i) a careful analysis of the genetic alterations coexisting with *MET* amplification and/or expression and (ii) an accurate identification of the genetically distinct subclones coexisting in the tumor. This may not be an easy task but technology will help in the near future (e.g., "next generation" sequencing, cell sorting, identification of "surrogate markers," and co-clinical trials in "xenopatients"; refs. 46–48). Looking forward to achieving these technological breakthroughs, two state-of-the-art approaches are currently available: combination of MET targeted therapy with either radiotherapy or anti-angiogenic agents.

MET Inhibitors, Radiotherapy, and Antiangiogenesis

We recently showed that doses of ionizing radiation commonly used for tumor radiotherapy induce *MET* transcriptional upregulation in glioblastomas and other tumor types (38). The signaling pathway leading to MET overexpression starts with ATM kinase, involved in recognition of DNA damage, and ends with the transcription factor NF-ºB that activates the *MET* promoter. Overexpression results in ligand-independent MET activation, sensitization to subliminal concentrations of HGF, protection against radiation-induced apoptosis, and, notably, induction of invasive growth. Vice versa, MET inhibition by small-molecule kinase inhibitors or monoclonal antibodies radiosensitizes tumor cells *in vitro*and *in vivo* (38). Interestingly, Joo and colleagues in their recent article showed that MET is induced by ionizing radiation in glioblastoma stem cells, and its inhibition counteracts their inherent radioresistance (24). Altogether, these findings strongly suggest that association of radiotherapy with MET inhibitors could increase the chance to eradicate the glioblastoma stem cell population (Fig. 1). The anti-VEGF antibody bevacizumab, targeting tumor

neoangiogensis, and currently approved as single agent for recurrent glioblastoma, has provided only a modest therapeutic benefit (49). Indeed, bevacizumab induces a transient tumor regression, almost invariably ending in resistance and progression. In most cases, the tumor regains the ability to form blood vessels, while in the remaining cases (up to 30%), the tumor relapses with a seemingly infiltrative and angiogenic-independent pattern (50). Although lack of standardized imaging criteria to define relapse makes these findings controversial, an invasive response likely takes place after blood vessel inhibition (reviewed in ref. 51). Increasing experimental evidence points to MET as a major culprit. Indeed, reduced blood influx leads to decreased intracellular oxygen concentration and *MET* transcriptional upregulation by HIF-1 (see above; ref. 27). Further in this line, a recent article proposes that the invasive response to bevacizumab may also arise from the reversal by bevacizumab of VEGF-dependent MET receptor inhibition (52). Although the role of stem cells in the response to bevacizumab remains unknown at this time, these findings predict that association of MET and angiogenesis inhibitors would be beneficial to prevent the tumor "proinvasive response" to blood deprivation.

Conclusions

Analysis of neurospheres and cells prospectively isolated from fresh tumors allowed to identify MET as a functional marker of glioblastoma stem cells. MET expression is significantly associated with specific genetic features, such as normal *EGFR* gene and*PTEN* inactivation, and with defined gene expression profiles (mesenchymal-proneural). Upon stimulation by its ligand, MET contributes to glioblastoma stem cell self-renewal, invasiveness, tumorigenesis, and radioresistance. Genetic heterogeneity of glioblastomas and neurospheres suggest that multiple stem cell subclones, with distinct genetic alterations and different expression of MET, coexist in the same tumor. These observations have important consequences for identification of patients that could benefit of targeted therapies against MET and/or other meaningful targets in glioblastoma and other tumors. MET involvement in resistance to ionizing radiation or antiangiogenic agents suggests that MET inhibition may be useful in combination with radiotherapy or antiangiogenic treatments such as bevacizumab.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C. Boccaccio, P.M. Comoglio

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Boccaccio

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Boccaccio, P.M. Comoglio

Writing, review, and/or revision of the manuscript: C. Boccaccio, P.M. Comoglio

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Boccaccio, P.M. Comoglio

Study supervision: C. Boccaccio, P.M. Comoglio

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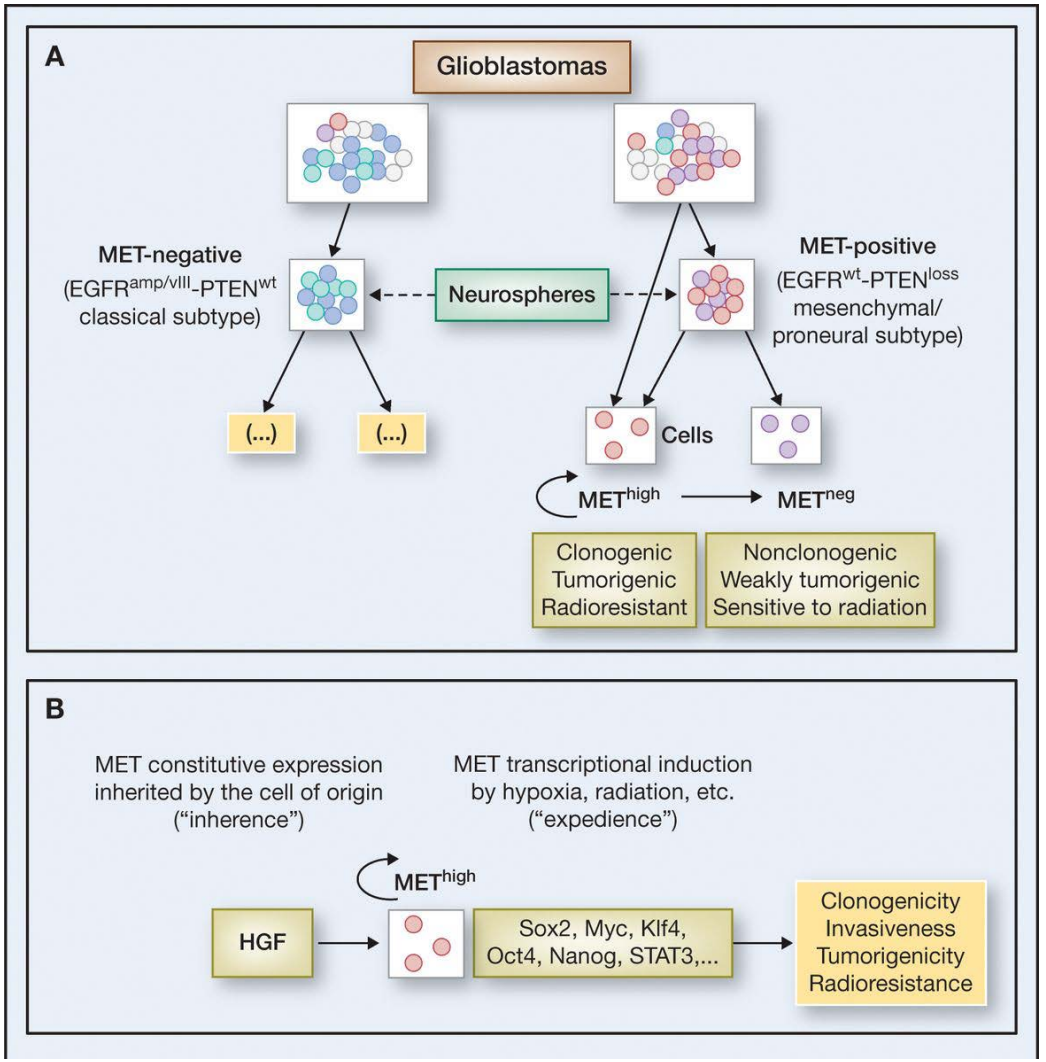
MET as a functional marker of glioblastoma stem cells.



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